

TAKAHASHI et al. S.N. 09/786,442

REMARKS

As noted above, applicants filed an amendment on April 9, 2003. The response included an excerpt from the publication "Antisense RNA and DNA" by James A.H. Murray. The excerpt consisted of 13 pages. However, applicants would also like to bring to the Examiner's attention three additional pages from that excerpt. Enclosed with this amendment are pages 12-14 from the Murray publication. In traversing the contentions of the Official Action, the Murray publication is discussed on page 17 in the amendment of April 9, 2003. Applicants note that the amendment of April 9, 2003 contained 35 pages (numbered 1-35). The excerpt from the Murray publication contains a total of 16 pages (cover, bibliographic page, pages 1-14). While the Murray excerpt contains page numbers 40-55. The Examiner is asked to disregard these page numbers as they are a result of a telefax from the applicant to the undersigned agent and do not reflect the page numbers of the article or response.

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In view of the amendment of April 9, 2003 and the present amendment, it is believed that the present application is now in condition for allowance.

Respectfully submitted,

YOUNG & THOMPSON

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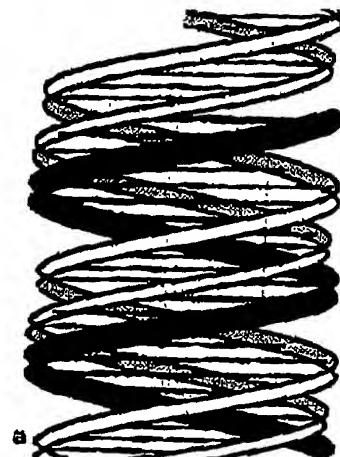
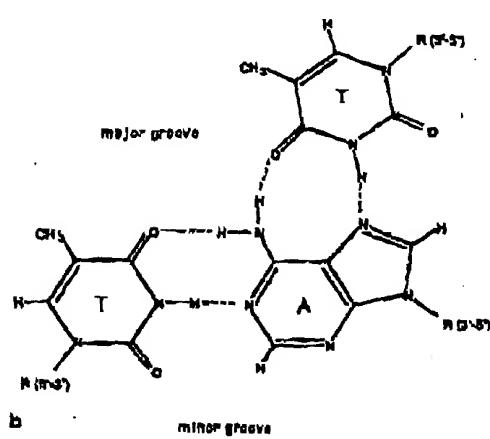
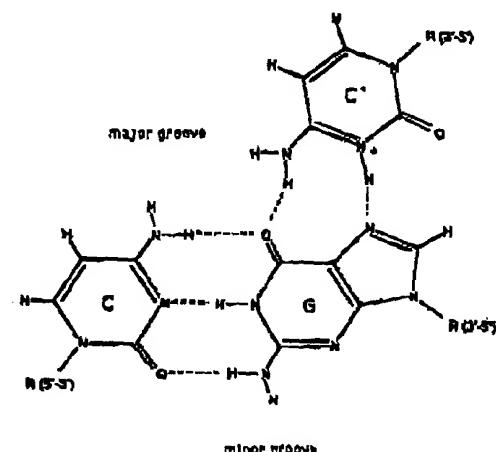


Fig. 3. a: Schematic generalized structure of a triple helix. The pyrimidine oligonucleotide (dark shading) is bound in the major groove of a Watson-Crick double helix parallel to the purine strand. b: Hydrogen bonding interactions involved in Watson-Crick base pairing (across) and in Hoogsteen triple helix formation (upwards). Hoogsteen hydrogen bonding of thymine to Watson-Crick A-T and protonated cytosine to G-C base pairs is shown. c: Example of triple helix-forming oligonucleotide. This sequence was used by Strobel and Dervan (1991) to block EcoRI methylation at a specific site in the yeast genome. Subsequent dissociation of the oligonucleotide and digestion with EcoRI resulted in cleavage only at the protected site, all other sites in the genome having been methylated. In this case oligonucleotides with C and T , ^{14}C and

T_c or MgC and ^{32}U were tested and were effective up to pH 7.4, 7.6, and 7.8, respectively. At "Switchback" or alternate-strand triple helix-forming oligonucleotide capable of binding parallel to both purine strands of a DNA duplex in the opposite orientation, as described by Horne and Dervan (1990).

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Antisense Techniques

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gene is targeted rather than the mRNA, since many copies of an mRNA are produced from a single equivalent of DNA. The steady-state level varies in lymphoid cells, for example, from about 10 per cell for rare mRNAs, to 200 copies for nonabundant, and up to 30,000 copies for each of the members of the "abundant class" [Schröder et al., 1989], and constant synthesis occurs. In contrast, most genes and proviruses are present at low number, often only one to two per cell, and DNA replication occurs only before cell division, which is far less frequent than the rate at which genes are transcribed into new mRNA molecules. In particular, the possibility of triple helix-forming oligonucleotides linked to cross-linking, alkylating, or cleavage reagents [Povsic and Dervan, 1989; Strobel and Dervan, 1990; Ferrouault et al., 1990] offers the prospect of long-term inactivation of target genes or proviruses at low therapeutic doses, since such agents may act to stabilize the triple helical binding and/or inactivate the DNA.

Although it has been shown *in vitro* that triple helix-forming agents can block enzymatic access to DNA by restriction enzymes or methylases [Francois et al., 1989; Maher et al., 1989; Hanvey et al., 1990; Strobel and Dervan, 1991] and by a eukaryotic transcription factor [Maher et al., 1989], there is only one example to date in which it has been speculated that regulation of gene expression under physiologic conditions may be attributable to triple helix formation [Cooney et al., 1988]. In this case, regulation of *c-myc* expression by a mixed-sequence oligonucleotide was observed, and a triplex structure was proposed but not proven, although supported by indirect evidence including the effectiveness of the oligonucleotide in the 100 nM range. The oligonucleotide sequence was purine rich, so Hoogsteen base pairing could not have been involved, and the molecular basis for the recognition has not yet been established. It does, however, raise the possibility that an alternative pattern of recognition of double-stranded DNA by purine oligonucleotides could exist, in addition to the established pyrimidine oligonucleotide Hoogsteen motif.

At present, however, a major limitation of the triple helix approach is the available code,

since it is restricted to the recognition of homopurine tracts by pyrimidine oligonucleotides. The runs of 15-18 purines that are required for a target site occur only rarely within genes that would be a potentially interesting target for regulation by triple helix formation, and, for the yeast experiments described above, the target was introduced by prior transformation. A partial way around this problem is the use of alternate-strand multizyme crossover oligonucleotides that produce so-called switch-back triple helix formation, which consists essentially of two short (8- to 9-mer) pyrimidine oligonucleotides joined at their 3' ends by a 3'-3' linkage. Such oligonucleotides are therefore capable of simultaneous binding to a purine segment on one strand and a second purine segment running in the opposite direction on the other strand. This was demonstrated by Horne and Dervan [1990] with the oligonucleotide illustrated in Figure 3d. This alternate-strand purine binding greatly increases the potential number of sequences that can be read to include those of the type 5'-(purine)_m-(pyrimidine)_n-3' and 5'-(pyrimidine)_m-(purine)_n-3', where m and n need be no greater than 8-9 bp.

However, it would clearly be desirable to extend the triple helical recognition code to a general solution in which all four base pairs of Watson-Crick double strands could be recognized at 37°C and physiological pH. Griffin and Dervan [1989] have shown that the code can formally be extended to the recognition of T-A base pairs by G within the environment of an otherwise pyrimidine oligonucleotide but that limitations on sequence composition are likely. The existence of other third-strand recognition interactions leading to G-G-C and A-A-T triplets has been explored by Letai et al. [1988], by binding to homopolymer agarose affinity columns, but the utility of these results to the recognition of mixed sequences has not been shown. Possibly more fruitful approaches may be the design of non-natural analogs to complete the triplex code or the incorporation of abasic residues that would allow certain Watson-Crick bases to remain unread.

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Further possibilities that remain to be explored are whether triple helix formation can also act to *activate* transcription by altering the local parameters of DNA conformation appropriately and the possibility of using analogs to improve the affinity or efficacy of binding. Hélène and coworkers [reviewed by Hélène and Toulmé, 1990] have shown that α -anomers of oligonucleotides can also form triple helical structures with normal double-stranded DNA, in which the phosphodiester backbone may have the opposite orientation to that of a β -oligomer. Despite current interest, much work is required to demonstrate the action of triple helix formation in cells in culture and *in vivo* and to define the parameters required for its action, including the response to DNA repair and replication, before it can be seriously considered as a therapeutic tool.

5. Oligonucleotides selected against protein targets. A recent approach [Riordan and Martin, 1991] has been the use of *in vitro* selection techniques to generate compounds with high affinity for biological target molecules in a manner analogous to that described by Joyce (this volume) for optimization of ribozyme function. In general terms it involves the use of the polymerase chain reaction (PCR) or other enzymatic techniques to amplify those oligonucleotides from a random pool that demonstrate affinity for the target molecule. The starting material is a pool of up to 10^{13} different oligonucleotide species, each with a different sequence. These are incubated with the target molecule or passed through an affinity column of the target, and bound oligonucleotides are separated from the unbound. The bound oligonucleotides with greater affinity for the target are then eluted and amplified and then subjected to further rounds of binding, elution, and amplification until the selection procedure results in an enriched pool that contains only those oligonucleotides with the highest binding affinity. In this way oligonucleotides are selected that have, by chance, the correct three-dimensional structure to bind to the target molecule under the selection conditions. It is then possible to test the ability of the selected oligonucleotide

to inhibit the biological activity of the target. Such oligonucleotides, dubbed "aptamers," have been generated to small organic molecules [Ellington and Szostak, 1990] as well as to large proteins, such as transcription factors [Blackwell and Weintraub, 1990; Blackwell et al., 1990] and T4 DNA polymerase [Tuerk and Gold, 1990].

D. Ribozymes

An extension of the antisense approach is to confer catalytic activity on the antisense RNA molecule such that cleavage of the target mRNA substrate occurs, resulting in its inactivation without affecting the antisense molecule. This is then free to dissociate and attack other substrate molecules, thus acting catalytically. In contrast, "conventional" antisense RNAs are required in stoichiometric amounts, since they must either remain hybridized to block mRNA function or target the duplex for degradation, in which case they are destroyed along with their substrate. The potential attraction of catalytic RNA systems for gene regulation or antiviral strategies is therefore that a lower concentration of antisense RNA molecules may be necessary for effective regulation.

RNA enzymes, or *ribozymes*, a term first used by Kim and Cech [1987], have been described in a number of naturally occurring systems and are responsible for cleavage and ligation of specific phosphodiester bonds within RNA molecules. Here we briefly review the main classes, concentrating on the progress that has been made on engineering them into catalytic antisense RNAs, i.e., enzymatic cleavage agents whose specificity is provided by antisense RNA sequences linked to the catalytic centre of the ribozyme. Further reviews are listed in Table I.

1. Group I introns: The *Tetrahymena* ribozyme. The first ribozyme activity to be discovered was that responsible for the autocatalytic removal of the intron from the precursor of the 26S ribosomal RNA of the ciliate *Tetrahymena thermophila*. In this and other introns (termed group I) excision of the intron and ligation of the exons to form the mature RNA occur as a result of specific folding of intron sequences